Macrophage Function in Mice with a Mutation at the Microphthalmia (*mi*) Locus

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Abstract. **Microphthalmic (*mi/mi*) mice are unpigmented, osteopetrotic, mast cell deficient, and exhibit diminished gastric acid secretion and natural killer cell activity. In order to assess the effect of this mutation on macrophage function, we studied superoxide (O_2^-) and nitric oxide (NO) production, superoxide dismutase (SOD) activity, phagocytic capacity, and tumor cell killing by peritoneal macrophages from *mi/mi* mice and normal (*+/+*) litter mates. Macrophages from *mi/mi* mice, upon activation with phorbol myristate acetate (PMA), generated significantly higher amounts of O_2^- than did macrophages from their *+/+* litter mates. The phagocyte respiratory burst as assessed by nitroblue tetrazolium (NBT) reduction assay also displayed a 2-fold enhancement in *mi/mi* macrophages. The assay of SOD activity revealed significantly lower levels in *mi/mi* macrophages than in the wild type. Furthermore, in comparison with controls, macrophages from *mi/mi* mice demonstrated significantly higher levels of NO production and increased capacity to lyse tumor cells upon activation with lipopolysaccharide (LPS) or gamma-interferon (IFN-γ). The *mi* mutation, therefore, is associated with reduced macrophage SOD activity, increased O_2^- and NO production, and enhanced capacity for tumor cell killing. The molecular mechanisms for these changes have not been identified.** [P.S.E.B.M. 1997, Vol 215]

The microphthalmia (*mi*) mutation in the mouse is associated with a number of alleles and variable phenotypes which include pigment abnormalities, microphthalmia, defective osteoclasts, deafness, and mast cell deficiency (1–4). Natural killer cell function may be normal or defective in *mi/mi* mice (4, 5), and the phagocytic capacity of macrophages is impaired (6). In addition, the *mi/mi* mice exhibit impaired gastric acid secretion (7) and bone resorption (6).

The *mi* gene, which disrupts the expression of a novel protein of the basic-helix-loop-helix-leucine zipper family of transcription factors, has been identified and cloned (8). It is unclear whether all of the abnormalities noted in the *mi/mi* animals are directly due to the defective nuclear transcription factor or are the consequences of impaired or altered communication between cells. Previous studies from our laboratory have shown that mast cells are capable of inhibiting a variety of macrophage functions such as superoxide (O_2^-) (9, 10) and nitric oxide (NO) production, tumor cell killing (11), and Fcγ-receptor–mediated phagocytosis (12). The absence of mast cells in *mi/mi* mice and the recognized interaction between mast cells and macrophages, along with the potentially broad effect of a transcription factor defect, prompted us to study the function of macrophages carrying the *mi* mutation. Evidence presented in this report indicates that macrophages from *mi/mi* mice produce significantly more O_2^- in response to PMA, more NO in response to LPS and IFNγ, and are more effective in tumor cell killing. The phagocytic capacity of *mi/mi* macrophages, as determined by the uptake of latex beads, was identical to normal controls. Macrophages from *mi/mi* mice also exhibited lower levels of superoxide dismutase (SOD) activity, which may partially explain the increased release of O_2^- and NO.

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Materials and Methods

Animals. The normal (*+/+*) and *mi/mi* mice used in these studies were 2- to 3-month-old males descended from C57Bl/6J *+/mi* breeders maintained at the University of Kansas Medical Center's animal care facility. The *mi/mi* mice were distinguished from their *+/+* litter mates by microphthalmia and pseudo-albinism. The heterozygotes (*+/–*) were distinguished from *+/+* animals by the presence of a large dorsal white patch that extended to the ventral surface. All animals were housed in microbarrier cages and were free from any known pathogens. Animals had free access to powdered food and purified water. The animal experimentation protocols presented in this study were approved by the Institutional Animal Care and Use Committee.

Isolation of Peritoneal Macrophages. Unless otherwise specified, elicited macrophages were collected 5 days after intraperitoneal injection (0.04 ml/g body wt) of a sterile solution of 4% thioglycollate (13). Resident cells were harvested from mice that did not receive any injections. The cells were collected by lavaging the peritoneal cavity of each animal with Tyrode's buffer containing 0.1% gelatin (TG) and 10 U/ml heparin (14). After pooling the cells and lysing the contaminant erythrocytes with Tris-buffered ammonium chloride, macrophages were isolated by centrifugation on 22.5% metrizamide (9–11). Macrophages separated at the interface were collected, washed twice, and resuspended in TG. Macrophages isolated in this manner exceeded 90% in purity and viability. Cell suspensions were used for the evaluation of phagocytosis, O_2^- production, and SOD activity. For experiments involving NO production and cytotoxicity assays, proteose peptone elicited-macrophages were harvested asceptically using minimum essential medium (MEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml heparin (11).

Superoxide Production.

Cytochrome c reduction assay. Superoxide production by macrophages was assayed by means of the SOD-sensitive reduction of ferricytochrome c with minor modifications (15, 16). The reaction mixture contained 3 × 10^6 macrophages, 50 nmoles of cytochrome c, and 6 μmoles of glucose in a final volume of 1 ml TG. The reagents were preincubated for 5 min at 37°C before activation of macrophages with PMA (0.1 or 1 μg/ml). The reaction was terminated after 5 min by chilling on ice followed by centrifugation. Supernatants of the reaction mixture were collected, and the absorbance was measured at 550 nm. The extinction coefficient of 21 mM^{-1} cm^{-1} was used to calculate superoxide production. The specificity of the assay was confirmed by adding excess amounts of commercial SOD (150 units) with each test. The SOD-sensitive reduction of cytochrome c was linear for at least 5 min after the challenge, and, therefore, 5-min assays were employed in these experiments.

Nitroblue tetrazolium (NBT) assay. The capacity of *+/+* and *mi/mi* macrophages to undergo respiratory burst was also evaluated by the NBT assay, which measures intracellular reduction of the dye to a blue formazan by O_2^-. In brief, 5 × 10^5 macrophages were incubated with NBT in 1.0 ml TG for 5 min and then stimulated with PMA (1 μg/ml). The cells were collected by centrifugation and solubilized with dioxane. The optical density of the solubilized formazan was measured at 580 nm (17, 18).

Phagocytosis Assay. Macrophage phagocytosis was studied by flow cytometric evaluation of the uptake of latex beads (19) with minor modifications. Macrophages (2 × 10^6) were incubated with fluorescent latex beads (0.9 μm size) in 1 ml TG for 15 min at 37°C. Following incubation, the cell suspensions were underlayed with 2.0 ml of 0.35 *M* sucrose, and the unphagocytosed beads were separated by centrifugation at 50*g* for 10 min. The sedimented macrophages were washed twice and resuspended in 1 ml TG. Phagocytosis of latex beads was quantitated with a fluorescence activated cell sorter (Model Epics 752; Coulter Electronics, Inc., Miami, FL).

Assay of Superoxide Dismutase. The superoxide dismutase (SOD) activity in disrupted macrophages was assayed by monitoring the scavenge of O_2^- generated by xanthine and xanthine oxidase as previously described (20, 21). Macrophages were sonicated in 50 m*M* potassium phosphate containing 0.5% Triton X-100 (pH 7.0) for 2 × 20 sec. The sonicates were centrifuged at 15,000*g* for 30 min at 4°C, and the supernatants were assayed for SOD activity. The assay system contained 50 m*M* potassium phosphate (pH 7.8), 0.1 m*M* xanthine, 0.02 m*M* cytochrome c, 0.1 m*M* EDTA, 4- to 5-μ units of xanthine oxidase, and macrophage extract in a final volume of 3.0 ml. The reaction was initiated by the addition of xanthine oxidase and the increase in absorbance was followed at 550 nm. SOD activity was determined by the degree of suppression of O_2^--mediated reduction of cytochrome c in the linear portion of the reaction. SOD activity is expressed as nanomoles of O_2^- scavenged.

Nitric Oxide Production. Macrophages originated from 2 × 10^5 peritoneal cells were allowed to adhere for 2 hr on each of the wells of a 96-well microtiter plate. The nonadherent cells were removed by washing, and the macrophage monolayers were stimulated with 1 μg/ml lipopolysaccharide (LPS) or 100 U/ml murine IFN-γ for 18 hr at 37°C (11, 22). Each set of treatments was run in quadruplicate. The production of NO by macrophages was determined by assaying the culture supernatants for nitrite content using Griess reagent (11, 22, 23).

Macrophage-Mediated Tumor Cell Killing. Macrophage-mediated cytotoxicity was measured using an 18-hr ^{51}Cr release assay as previously described (11, 22). In brief, P815 mastocytoma target cells, free of mycoplasma contamination, were labeled with ^{51}Cr by incubating at 37°C for 80 min. The cells were washed three times, and resuspended in minimum essential medium containing 15 m*M* HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), and fetal bovine serum (10% v/v) (HMEM). The cells were then centrifuged and adjusted to a suitable concentration. To assay cytolytic activity, macrophage monolayers (2×10^5) were incubated for 4 hr with various doses of LPS in 0.1 ml HMEM. After the preincubation, 2×10^4 ^{51}Cr-labeled P815 cells were added into each well in 0.1-ml aliquots. Eighteen hours later, the uppermost 0.1 ml of supernatant was collected and assayed for radioactivity in a gamma counter. Each experimental point is the result of quadruplicate assays. Results were expressed as percent specific ^{51}Cr release.

Results

Effect of the *mi* Gene on the Peritoneal Macrophage Population. The absolute numbers of resident and elicited peritoneal macrophages were decreased in the *mi/mi* mice when compared with normal litter mates (*+/+*). The mutant animals also had significantly lower body weights (*+/+*, 29.0 ± 1.3 g versus *mi/mi*, 14.1 ± 0.4 g; $n = 16$). However, when the cell numbers were adjusted for body weight, the *mi/mi* mice had more resident and elicited peritoneal macrophages (Table I). Both groups of animals showed a comparable time course in responding to thioglycollate elicitation and had more than a 10-fold increase in macrophage numbers. The elevated numbers of elicited macrophages remained for more than 7 days in both groups.

Macrophage Phagocytosis. In order to evaluate the phagocytic capacity of macrophages, equal number of cells were incubated with fluorescent latex beads, and the phagocytosed beads were quantitated using flow cytometry. The fluorescence intensity curves for *mi/mi* and *+/+* mice were identical when equal numbers of peritoneal macrophages were incubated with fluorescent latex beads and analyzed by flow cytometry (Fig. 1). Although it was impossible to be certain whether a given bead was actually phagocytosed or merely viewed overlying a macrophage, the sucrose density gradient centrifugation and vigorous washing should have removed the beads that were not attached to or within the cells. Hence, the fluorescence intensity could be attributed to adhered or internalized beads.

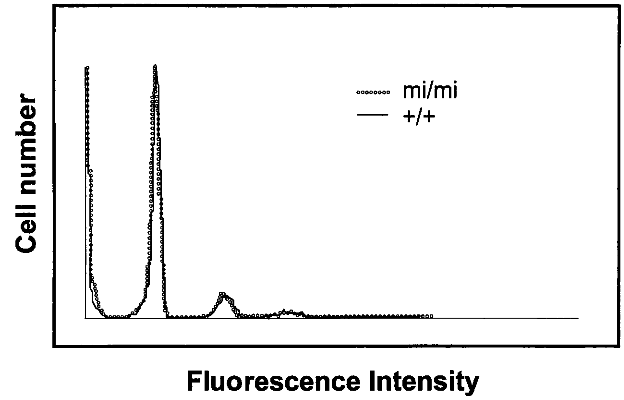

Figure 1. Flow cytometric analysis of phagocytosis of fluorescent latex beads by peritoneal macrophages as demonstrated by the fluorescence intensity plotted against cell number. The experimental details are given in Materials and Methods. Histograms of fluorescence intensity for *mi/mi* and *+/+* are superimposed for comparison.

Table I. Number of Resident and Elicited Peritoneal Macrophages in *+/+* and *mi/mi* Mice (Cell Number × 10^{-3}/g Body Wt)

Genotype	Resident	Elicited (days after thioglycollate injection)			
		4	5	6	7
+/+	92 ± 5	1380	560	480	300
mi/mi	139 ± 9[a]	1460	660	600	350

Note. Macrophages were collected by lavage of the peritoneal cavity of naïve or thioglycollate-injected mice as described in Materials and Methods. Values given for resident macrophages are mean ± SEM of three separate experiments consisting of three to five animals per group. Values given for the elicited macrophages are from a single experiment consisting of pooled cells from three to five animals per time point. The body weights of *+/+* and *mi/mi* mice are 29.0 ± 1.3 and 14.1 ± 0.4 (mean ± SEM; $n = 16$), respectively.

[a] $P < 0.05$ when compared with *+/+*.

Superoxide Production. Superoxide production is a vital macrophage function for bacterial killing and tumor cell lysis. In order to assess the effect of the *mi/mi* allele on O_2^- generation, we compared macrophages from *mi/mi* and normal litter mates. Superoxide production by *mi/mi* macrophages, as measured by cytochrome c reduction, was close to double that of normal resident macrophages at 0.1 and 1.0 μg/ml of PMA (Table II). Superoxide production by macrophages was further evaluated using an NBT reduction assay. Again, the *mi/mi* macrophage produced twice the reduction of NBT as compared with the macrophages from *+/+* mice. Elicited macrophages from animals with the *mi* allele showed a similar 2-fold increase in superoxide production when the cells were harvested at Day 3 and Day 7.

Superoxide Dismutase Activity. Superoxide dismutase plays a key role in the homeostasis of reactive oxygen metabolism because of its ability to scavenge O_2^-. The observed increase in O_2^- production in *mi/mi* macrophages may be due to an increased respiratory burst, a decrease in the cellular SOD activity, or a combination of both. In this study we assayed SOD activity in *+/+* and *mi/mi* macrophage extracts. The O_2^- scavenging ability of the cell extract from *mi/mi* animals was less than half of that from normal macrophages (Fig. 2). This relationship held true for extracts obtained from both resident and elicited macrophages.

Nitric Oxide Production. In an 18-hr culture, macrophages from *mi/mi* mice produced significantly greater amounts of NO upon activation with LPS or IFN-γ (Fig. 3). Neither the *mi/mi* nor the *+/+* macrophages produced detectable amounts of NO in the absence of activators.

Macrophage-Mediated Tumor Cell Killing. Macrophage-mediated tumor cell lysis may be dependent on multiple factors including O_2^- and NO. In view of the increased production of O_2^- and NO by mutant macrophages, the effectiveness of these macrophages were assessed for cytotoxicity. As shown in Figure 4, macrophages from *mi/*

Table II. Superoxide Production by Resident Peritoneal Macrophages from *+/+* and *mi/mi* Mice

Genotype	Cytochrome c reduction (nmoles/min/10^6 cells)		NBT reduction (μg of formozan formed/min/10^6 cells)
	0.1 µg PMA	1.0 µg PMA	1.0 µg PMA
+/+	0.71 ± 0.07	1.05 ± 0.03	0.17 ± 0.03
mi/mi	1.40 ± 0.17[a]	2.51 ± 0.65[a]	0.38 ± 0.03[a]

Note. Superoxide production by PMA-activated macrophages was determined by SOD-sensitive reduction of cytochrome c and by quantifying NBT reduction as described in Materials and Methods. Each value given is the mean ± SEM of four separate experiments.

[a] $P < 0.01$ when compared with *+/+*.

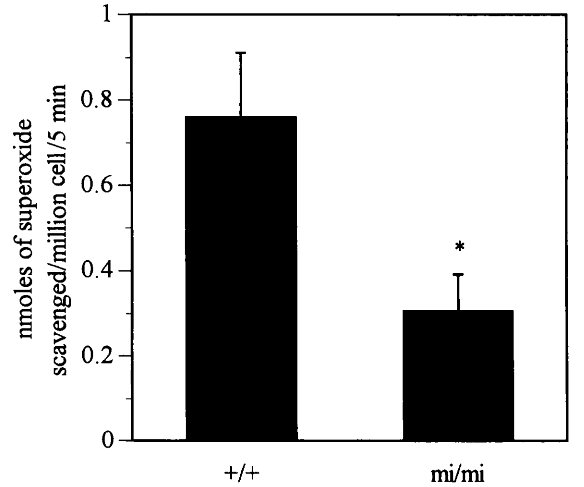

Figure 2. Superoxide dismutase activity in peritoneal macrophages from *+/+* and *mi/mi* mice. Macrophages were sonicated in 50 m*M* potassium phosphate containing 0.5% Triton X-100 (pH 7.0) for 2 × 20 sec. The sonicates were centrifuged at 15,000*g* for 30 min at 4°C and the supernatants were assayed for SOD activity by monitoring their ability to scavenge superoxide generated by xanthine and xanthine oxidase. The results presented are the mean ± SEM of six experiments in each group, with each experiment done using pooled cells from three to five mice. $^*P < 0.05$ when compared with *+/+*.

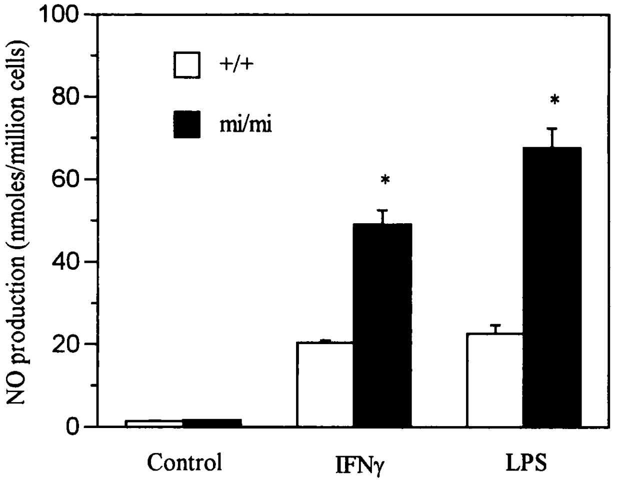

Figure 3. Nitric oxide production by peritoneal macrophages from *+/+* and *mi/mi* mice. Adherent macrophages originated from 2 × 10^5 peritoneal exudate cells were cultured for 18 hr with IFN-γ (100 U/ml) or LPS (1 µg/ml) as described in Materials and Methods. NO production was assayed by quantifying nitrite content in the culture supernatants. The data presented are the mean ± SEM of quadruplicate determination from a typical experiment. Similar results were obtained in two other separate experiments. $^{**}P < 0.01$ when compared with *+/+*.

mi mice exhibited significantly higher cytolytic activity towards P815 mastocytoma cells than macrophages from the *+/+* litter mates when activated with LPS. The enhanced cytotoxicity of *mi/mi* macrophages was evident at all doses of LPS tested.

Discussion

Mast cells have been implicated in the amplification of a nonspecific inflammatory response in rat skin (24). Although the *mi/mi* animals are mast cell deficient, macrophage recruitment to the peritoneal cavity in *mi/mi* mice is normal both in the naïve state or after thioglycollate injection (Table I). The *mi* mutants are smaller in size than their normal litter mates and have a lower absolute number of peritoneal cells. However, on body weight basis, the cell numbers in *mi/mi* mice are comparable to the normal controls. When the numbers of resident or elicited peritoneal macrophages were compared, *mi/mi* mice had greater or equal number of cells based on a unit body weight. This is in contrast to the previous report showing subnormal numbers of elicited peritoneal macrophages in *mi/mi* mice (6). As the *mi/mi* fails to grow and gain weight compared with the *+/+* litter mate, absolute numbers of any type of cell would be expected to be proportionately decreased. Therefore, the assessment of cell numbers on a body weight basis is more appropriate.

Previous studies have reported that adherent macrophages from *mi/mi* mice have impaired phagocytosis of latex beads (6). Our experiments utilizing macrophages in suspension did not demonstrate any defect in the phagocytosis of latex beads as assessed by flow cytometry (Fig. 1). Adherent cells may be partially activated, and this may uncover a subtle phagocytic defect not observed with cells in suspension. This is consistent with the normal handling of staphylococcal organisms and glutaraldehyde-fixed red cells reported in the earlier study (6). In the present study, the phagocytic capacity of *mi/mi* macrophages as determined by the uptake of fluorescent latex beads was identical to that of *+/+* mice. The reported deficiency in latex bead phagocytosis may be related to experimental design. The *mi/mi* macrophages have difficulty adhering to glass and obtaining a typical ruffled morphology (6). This could result

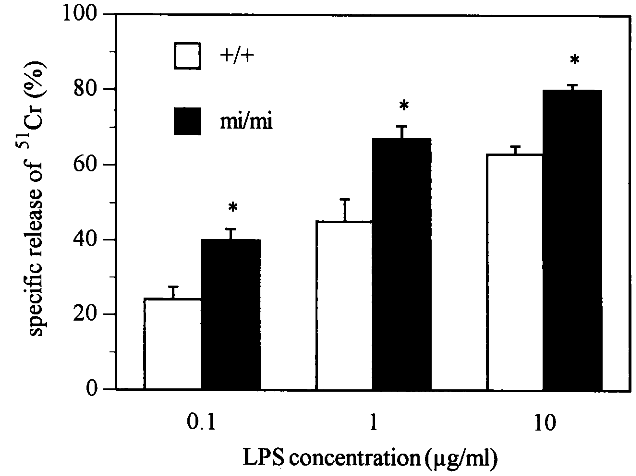

Figure 4. Tumor cell killing by peritoneal macrophages from *+/+* and *mi/mi* mice. Adherent macrophages originated from 2×10^5 peritoneal exudate cells were activated with the indicated doses of LPS and were cultured for 18 hr with ^{51}Cr-labeled P815 cells (2×10^4) as described in Materials and Methods. Tumor cell killing was assayed by monitoring ^{51}Cr release. Each value is the mean ± SEM of quadruplicate determinations. *$P < 0.01$ when compared with *+/+* for each LPS dose tested.

in diminished surface area and a decrease in the probability that a given latex bead would come in contact with a macrophage membrane. In our experiments, the macrophages were incubated in suspension and not allowed to adhere to a surface. Therefore, the surface areas of the cells should have been comparable.

Macrophages from mutant animals exhibited a consistent increase in superoxide production in response to PMA challenge, as determined by O_2^--mediated cytochrome c reduction and NBT reduction. Superoxide production by the phagocytic cells is initiated by the membrane-bound respiratory burst enzyme, NADPH oxidase. NADPH oxidase is dormant in resting phagocytes, and its activation by external stimuli involves translocation and assembly of cytosolic components to the plasma membrane (25–27). Superoxide dismutase plays a key role in the homeostasis of reactive oxygen radicals as this enzyme scavenges O_2^-. Thus, the net amount of O_2^- output is the balance between NADPH oxidase and SOD activity. The evidence presented here demonstrates decreased SOD activity in *mi/mi* resident and elicited macrophages. Thus, the reduced SOD activity may be a dominant causative factor for the elevated output of O_2^- in *mi/mi* mice. It is noteworthy that mast cells are rich in SOD (10, 21, 28), and interaction of mast cell granules with macrophages *in vitro* results in reduced O_2^- output by macrophages (9, 10). This phenomenon may also occur *in vivo* in normal animals but would be lacking in mast cell–deficient animals.

The *mi* mutation has been reported to affect the production of a basic-helix-loop-helix-leucine zipper transcription factor (8). The present study demonstrates that the *mi* allele is associated with reduced SOD activity (Fig. 2) and increased generation of superoxide (Table II) and NO (Fig. 3). These findings were evident in both resident cells and elicited cells harvested at 3 and 7 days after thioglycollate injection. The decreased level of SOD activity in the *mi* mutant macrophages compared with the normal cells (Fig. 2) could result in reduced dismutation of O_2^-. The effect of this enzyme on NO production and release is unclear. Conversely, in transgenic mice with overexpression of SOD, macrophage production of O_2^- and NO were reduced (29). The decreased level of SOD in *mi/mi* macrophages could either be due to the direct gene effect or the absence of mast cells, which would eliminate any possible contribution of mast cell SOD to the macrophages. Further studies are required to define the effect of *mi* mutation on the SOD gene. The effect of the *mi* mutation on NADPH oxidase also remains to be evaluated.

Macrophage-mediated tumor cell killing is enhanced in *mi/mi* animals (Fig. 4). This is consistent with the increased production of both NO and O_2^- which are known participants in this phenomenon. Our previous studies have demonstrated a dose-dependent decrease in macrophage-mediated cytolysis of P815 cells when macrophages were co-incubated with mast cell granules, which was associated with decreased NO and O_2^- generation (9–11).

Previous studies indicate that the helix-loop-helix-leucine-zipper transcription factor is defective in *mi* mutants (8). An impairment in the generation of O_2^- and NO has been demonstrated in transgenic mice expressing high levels of CuZn-SOD (29). The current study supports the inverse relationship between SOD activity and the production of O_2^- and NO in macrophages. Furthermore, this is the first demonstration that the *mi/mi* allele is associated with reduced SOD activity. Additional studies are warranted to determine whether altered *mi/mi* macrophage functions are exclusively limited to the gene effect on this cell.

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